Characterization and measurement of human apolipoprotein A-II by radioimmunoassay

Ronald B. Goldberg,[1] Joan B. Karlin,[2] Dawn J. Juhn, Angelo M. Scanu, Celina Edelstein, and Arthur H. Rubenstein[3]

Departments of Medicine and Biochemistry, The University of Chicago Pritzker School of Medicine, Chicago, IL 60637

Abstract The development of a radioimmunoassay for apolipoprotein A-II (apo A-II) is described. Initial studies revealed a lack of immunological identity between purified apo A-II used as the standard and serum or HDL. Extensive testing of different buffers, standards, antisera, tracers, utilization of a detergent, and heating of sera failed to resolve the problem. Gel filtration of iodinated and non-iodinated apo A-II on Sephadex G-100 columns showed that apo A-II, in dilute solution, elutes in a higher molecular zone than expected with a broad, assymetrical profile. The use of a subfraction of the tracer in the assay resulted in parallelism in the serum and standard dilution curves. The apo A-II assay was sensitive, specific, and reproducible. Apo A-II added to sera was fully recovered and delipidation did not affect the immunoreactivity of either serum or HDL. Apo A-II contributed approximately 20% to the protein mass of HDL. Comparison of these results with those obtained by radial immunodiffusion, and with previously reported data, indicates that the reactivity of apo A-II in its native and delipidated forms may be markedly influenced by different immunologic methodologies and their specific reagents. Caution should thus be shown at present in assigning absolute concentrations to apo A-II in serum or HDL.—**Goldberg, R. B., J. B. Karlin, D. J. Juhn, A. M. Scanu, C. Edelstein, and A. H. Rubenstein.** Characterization and measurement of human apolipoprotein A-II by radioimmunoassay. *J. Lipid Res.* 1980. **21:** 902–912.

Supplementary key words high density lipoprotein

High density lipoproteins (HDL) have attracted increasing interest in recent years following the finding of an inverse correlation between coronary artery disease and HDL levels (1). HDL consists of approximately 50% lipid and 50% protein: the protein moiety contains two major polypeptides, apolipoprotein A-I (apo A-I) and apo A-II, and several minor components. Precise quantitation of the apoproteins is required in order to assess their role in HDL structure and function, and immunoassays are being developed for this purpose. Since immunological methods are sensitive to the structural form of the peptide being measured, variation in their molecular conformation may affect quantitation. Purified apo A-I and A-II commonly used as assay standards, have unique physicochemical properties (2–4) which may be different from those of the apoprotein when complexed with lipid. Hence, if antigenic sites are modified during the purification of the apoprotein, the immunoreactivity may be changed. This has been clearly demonstrated to be the case for apo A-I (5, 6) and was the basis for those difficulties experienced in developing an immunoassay for this apoprotein (6, 7).

Wide discrepancies in serum values of apo A-II have been reported (8–12),[4] which may reflect similar problems in the quantitation of this peptide. It was therefore important, in developing a quantitative immunoassay procedure for apo A-II, to characterize the assay components in some detail. The radioimmunoassay technique represents a powerful tool for accomplishing this goal, since, in addition to quantitation, this method provides a qualitative immunological assessment of the peptide being measured in relation to the standard. In this study, we describe a specific and sensitive double-antibody radioimmunoassay method for measuring apo A-II in which considerable attention has been devoted to these factors.

MATERIALS AND METHODS

Isolation of apo A-II

HDL (d 1.063–1.21 g/ml) was isolated by ultracentrifugation of fresh serum taken from fasting,

Abbreviations: HDL, high density lipoprotein d 1.063–1.21 g/ml; apo A-II, apolipoprotein A-II; apo A-I, apolipoprotein A-I; VLDL, very low density lipoprotein d < 1.006 g/ml; LDL, low density lipoprotein d 1.019–1.063 g/ml; HDL_2, high density lipoprotein d 1.063–1.12 g/ml; HDL_3, high density lipoprotein d 1.12–1.21 g/ml; TMU, 1,1,3,3-tetramethylurea; SDS, sodium dodecyl sulfate.

[1] Address reprint requests to Dr. Ronald B. Goldberg. Present address: Department of Medicine, University of Miami School of Medicine, R-760, Miami, FL 33101.

[2] Present address: Department of Medicine, Baylor College of Medicine, Texas Medical Center, Houston, TX 77030.

[3] Arthur H. Rubenstein is an Established Investigator of the American Diabetes Association.

[4] Bronzert, T. J. Personal communication.

healthy, normolipidemic male donors (13). Delipidation of HDL was carried out at −10°C with ethanol–ether 3:2 (v:v) and the apo HDL was fractionated into its major polypeptide components by Sephadex G-200 column chromatography in 8 M urea (13). Peak IV, containing apo A-II, was further purified after lyophilization by DEAE cellulose ion-exchange column chromatography in 6 M urea. Fractions corresponding to apo A-II were extensively dialyzed against 0.01 M NH_4HCO_3 and lyophilized. In some cases the final lyophilization step was omitted. The purity was checked by polyacrylamide gel electrophoresis (PGE) (14) in sodium dodecyl sulfate (SDS), by immunoprecipitation, and by amino acid analysis. Protein concentration was measured by the method of Lowry et al. (15); 1.0 mg of apo A-II estimated by this method was equivalent to 0.96 mg measured by amino acid analysis. Reduced-and-alkylated apo A-II was prepared using iodoacetamide as previously described (16).

Storage of apo A-II

Several methods of storage of purified apo A-II were assessed. These included i) freezing at −20°C in 10- or 20-μg aliquots dissolved in 0.01 M NH_4HCO_3 so that the sample is thawed once only; ii) freezing at −60°C in 0.01 M NH_4HCO_3; iii) freezing at −60°C in 1.5 M quanidine-HCl; iv) freezing at −60°C in the presence of 0.5% bovine serum albumin (fraction V); v) freezing at−60°C at pH 6.0; vi) freezing a larger amount (ca. 1.0 mg) of peptide in 0.01 M NH_4HCO_3 at −20°C with repeated freezing and thawing; and vii) lyophilization and storage at −60°C.

Iodination of apo A-II

Apo A-II was iodinated by a modification of the method of Freychet and Roth (17), essentially as described by Schonfeld and Pfleger (6), with the difference that the reaction was stopped by the addition of 5 μl of sodium metabisulfite (0.2 mg/ml). In some cases the lactoperoxidase method was used (18). The labeled peptide (sp act 36–58 mCi/mg) was separated from unreacted ^{125}I by gel filtration on 0.8 × 10 cm Sephadex G-75 (Superfine) columns equilibrated in borate–0.5% albumin buffer, pH 8.0 (7). Iodinated apo A-II was stored at 4°C or frozen in aliquots at −20°C. The iodinated tracer was used for a maximum of 4 weeks.

Preparation of antisera

New Zealand white rabbits or guinea pigs were immunized with either purified apo A-II, glutaraldehyde-complexed apo A-II, or human HDL (d 1.063–1.21 g/ml) mixed with complete Freund's adjuvant and injected into multiple intradermal sites. Animals with significant titers at 6 weeks were given a booster dose using incomplete Freund's adjuvant and were then bled 2 weeks later.

The radioimmunoassay

The assay was carried out using a 0.1 M borate buffer (pH 8.0) containing 0.5% bovine serum albumin (7). Both standard and sample dilutions were stable for at least 24 hr at 4°C. Standards or samples were made up to a total volume of 1.0 ml; 0.1 ml of ^{125}I-labeled apo A-II (20,000 cpm) and 0.1 ml of appropriately diluted antiserum were added to give approximately 50% binding of the tracer in the absence of added apo A-II. After incubation at 4°C for 3 days, 0.1 ml of goat antirabbit IgG (1:30) and rabbit serum as carrier (1:100) were added. After a further 48 hr incubation at 4°C, the tubes were centrifuged (520 *g*) at 4°C for 20 min. The supernatants were decanted and the precipitates counted. Results were expressed as B/Bo, where B = ^{125}I-labeled apo A-II cpm precipitated−cpm precipitated in non-specific binding tubes, and Bo = ^{125}I-apo A-II cpm precipitated in the zero dose tubes−cpm precipitated in non-specific binding tubes. Serum and standard displacement curves were subjected to log-logit transformation for some of the analyses [logit (B/Bo) = $\log_n$ (B/Bo/1-B/Bo)] and the data were fitted by the least squares method to a straight line. In all cases the correlation coefficients of the data were greater than 0.97.

Lack of parallelism between serum and standard displacement curves was evident when the serum value measured from data points along the serum dilution curve showed a changing (downward) trend in proportion to increasing amounts of serum put into the assay. Statistically this was verified by the fact that the slope of the serum displacement curve transformed by logit transformation lay outside the mean ± 2 SD of the slope of the standard.

Treatment of samples prior to assay

Samples were delipidated with ethanol–ether as previously described (19). In addition, some samples were treated with 8.4 M redistilled tetramethylurea (TMU) in 8 M recrystallized urea (8). Lipoprotein fractions were obtained by preparative ultracentrifugation (13). In addition, serum was fractionated by the single-spin density gradient ultracentrifuge technique previously described, modified to partially resolve HDL_2 from HDL_3 (20).

Quantitative polyacrylamide gel electrophoresis

Quantitative gel electrophoresis of four different HDL_3 preparations was carried out according to the method of Weber and Osborn (14) in 7.5% polyacrylamide containing 1% SDS. An apo A-II cali-

TABLE 1. Lack of parallelism between standard and serum displacement curves using anti-HDL and anti-A-II antisera

Antiserum	Slope of Standard (±2 SD)	Mean (±S.E.M.) Slopes of Sera
Rabbit anti HDL (G1)	−2.52 ± 0.20	−2.13 ± 0.03 (5)
Rabbit anti HDL (L-3)	−2.49 ± 0.18	−1.81 ± 0.05 (3)
Guinea pig anti HDL (809)	−2.50 ± 0.14	−2.07 ± 0.09 (3)
Rabbit anti A-II (Ra IV)	−2.18 ± 0.12	−1.93 ± 0.03 (3)

The slopes of the points on the standard displacement curve (±2 SD) were calculated from the logit transformation of B/Bo. The slopes of serum curves are expressed as the mean ± S.E.M. of the slopes of several sera displacement curves for each antiserum. The slope of each serum displacement curve differed by more than 2 SD from the slope of the standard. The numbers in parentheses indicate the number of serum samples tested.

bration curve was prepared by applying 5–15 μg of pure apo A-II to a series of gels. Dye staining of the apo A-II bands was quantitated using a Gilford spectrophotometer equipped with a linear gel scanner and recorder.

Storage of serum samples

Sera were stored either frozen or lyophilized at −20°C. The effect of repeated freezing and thawing was tested by subjecting fresh sera to seven freeze and thaw cycles over a 2-day period.

Quality control

Three quality control sera are included in each assay. Aliquots (50 μl) were stored either frozen or lyophilized at −20°C. Statistical analyses were performed according to standard methods (21). Values are expressed as the mean ± S.E.M.

Subjects

Control healthy normolipidemic subjects included hospital personnel and medical students who were within 15% of ideal body weight (22). There were 28 males and 22 females (ages 25–35 years). They did not give a family history of diabetes or hyperlipidemia.

RESULTS

Development of the APO A-II radioimmunoassay

Displacement of ^{125}I-apo A-II by standard and sera dilutions. The standard curve using the anti-HDL antiserum G1 showed significant displacement at 0.5 ng apo A-II. Non-specific binding ranged from 4.5–5.5% and at a final antiserum titer of 1:20,000, the working range of the standard lay between 1–10 ng of apo A-II (B/Bo 0.85–0.40). Maximal displacement of the tracer occurred between 500–1000 ng. However, dilutions of sera were usually not parallel to the standard. Several anti-HDL and anti A-II antisera were tested, and all were associated with lack of parallelism between serum and standard displacement curves (**Table 1**). Log-logit transformation of representative curves using the G1 anti-HDL antiserum are shown in **Fig. 1.** Although the lack of parallelism appears slight, values of three sera tested in seven different assays read at B/Bo = 0.5, were 24 ± 2.1% (range 7–44%) lower than when read at B/Bo = 0.8, and 28 ± 3.1% (range 8–44%) lower at B/Bo = 0.3.

Modifications of the assay. The use of several different buffers, varying the BSA concentration, substitution of gelatin for BSA, addition of 0.01% Triton X-100 to the buffer, and heating of serum dilutions (7) were tested, but did not improve the lack of parallelism between the sera and standards. When the HDL antiserum G1 was used at higher concentration (1:5000), the working range of the assay was shifted 10-fold (10–100 ng), but this did not improve the results. Four different purified apo A-II standard preparations were tested, but their dilution curves were not parallel to serum curves.

Gel filtration chromatography of apo A-II. **Fig. 2** illustrates the gel filtration elution profiles of an iodinated and an unlabeled apo A-II preparation applied separately to a 1.2 × 50.0 cm Sephadex G-100 column equilibrated in borate–0.5% BSA, pH 8.0. Ten μg of unlabeled apo A-II was applied and each fraction assayed for its immunoreactivity as described (recovery = 73%). Both unlabeled and iodinated apo A-II eluted with broad asymmetrical peaks originating in the void volume of the column. Most of both the apo A-II immunoreactivity and radioactivity eluted ahead of the 27,000 molecular weight ^{125}I-labeled apo A-I marker. The complexity of the elution profiles is contrasted with the homogeneity of these preparations when subjected to SDS acrylamide electrophoresis (Fig. 2). Five different apo A-II preparations showed similar gel filtration characteristics. Preincubation of the unlabeled apo A-II for 24 hr with 2 M guanidine HCl or iodination of apo A-II by the gentler lactoperoxidase method, did not alter the elution profiles.

Reduced-and-alkylated apo A-II. Since the apo A-II standard curve was not parallel to serum dilution curves, reduced-and-alkylated apo A-II was tested as an alternative standard. The dilution curve of this reduced apo A-II standard was also not parallel to serum dilution curves using ^{125}I-labeled reduced-and-alkylated apo A-II as tracer. Moreover serum values were approximately half those obtained with the non-reduced apo A-II system. Non-reduced apo A-II showed a similar decrease in immunoreactivity when ^{125}I-labeled reduced apo A-II was the tracer.

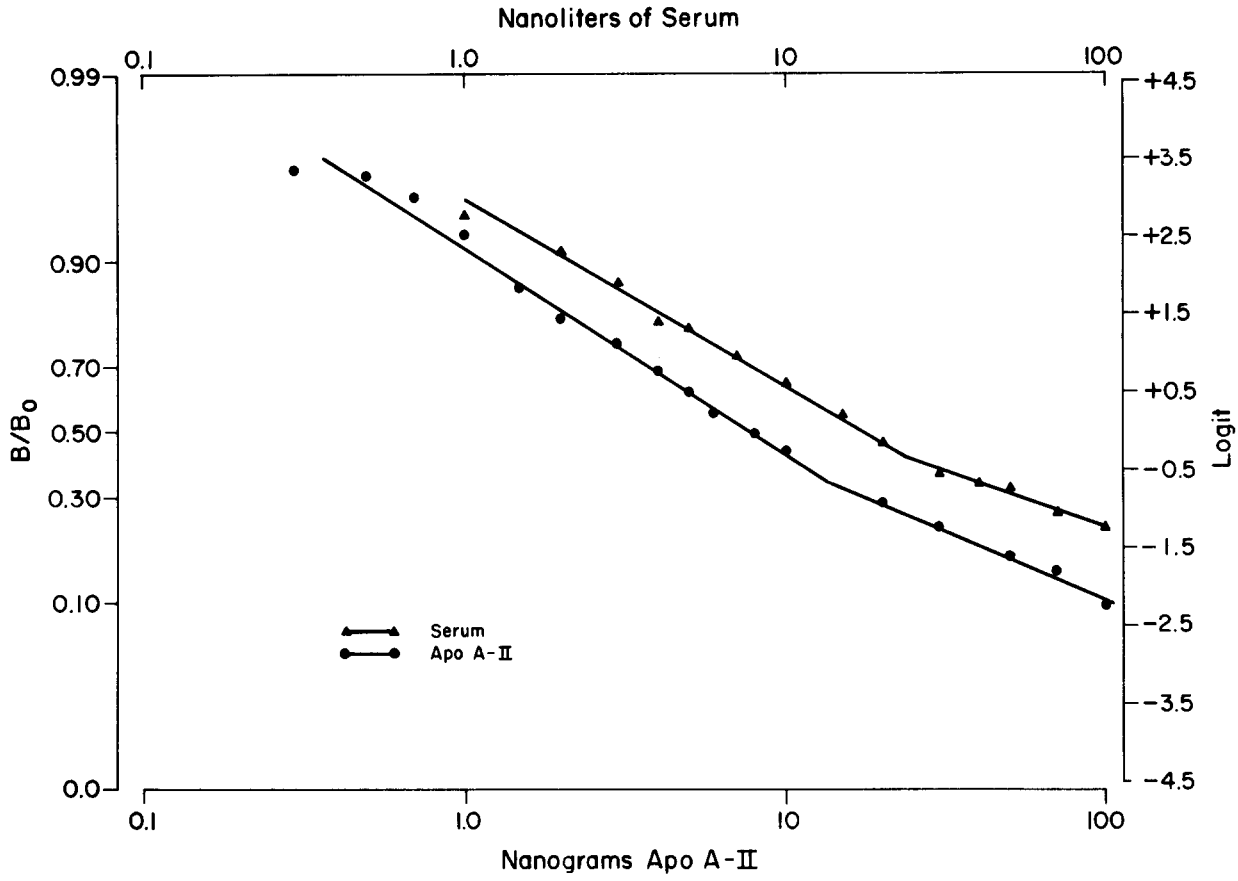

Fig. 1. Logit transformation of the displacement curves (B/Bo) of apo A-II (● —— ●) and serum (▲ —— ▲). The logit plot converts the standard curve to a straight line. The logit value is shown on the right hand ordinate and is 0 at B/Bo = 0.5. The bend in the line denotes the presence of two classes of antibody binding sites. The tracer was ^{125}I-labeled apo A-II and an HDL antiserum was used (G1).

Further purification of ^{125}I-labeled apo A-II. Freshly iodinated apo A-II was applied to a Sephadex G-100 column. The profile (Fig. 2) revealed a void volume peak (Peak I), a second peak in the column volume eluting before the ^{125}I-labeled human apo A-I marker (Peak II), and a third smaller component in the position of the 9,000 molecular weight marker (Peak III). Material from each of these three zones was reapplied to Sephadex G-100 columns (**Fig. 3**). Peak I material eluted entirely within the void volume. When this same fraction was applied to a Biogel A 0.5 M column equilibrated in borate–albumin buffer, a broad asymmetrical peak was again obtained, originating in the void volume of the column and extending well into the column volume.

^{125}I-labeled Peak I and -Peak II both migrated as single bands in the position of human apo A-II on SDS polyacrylamide gel electrophoresis. In the presence of β mercaptoethanol, a faster migrating band corresponding in position to reduced human apo A-II was observed. In contrast, ^{125}I-labeled Peak III migrated in the position of reduced A-II, whether preincubated with β mercaptoethanol or not.

Radioimmunoassay of human apo A-II using ^{125}I-labeled apo A-II subfractions. The displacement of ^{125}I-labeled apo A-II Peaks I, II, and III, as well as the unfractionated ^{125}I-labeled apo A-II used in the initial studies, by unlabeled apo A-II, was tested in the radioimmunoassay. While the Peak II tracer reacted similarly to the unfractionated ^{125}I-labeled apo A-II, the Peak I component was associated with a shift of the standard curve to the left. Both Peak I and II tracers resulted in assays in which there was parallelism between serum and standard curves and similar serum values (for three sera, Peak I: 34, 22, 28 mg/dl; Peak II: 31, 24, 30 mg/dl). In contrast, the Peak III tracer required a higher antiserum concentration in the assay and was not associated with parallelism between standard and serum dilutions.

The Peak I void volume component of the tracer was chosen for routine use as the assay tracer for the following reasons: i) it consistently provided parallelism between the standard and sera; ii) it migrated as a single band corresponding to unlabeled apo A-II on polyacrylamide gel electrophoresis; iii) it comprised the most clearly defined portion of the un-

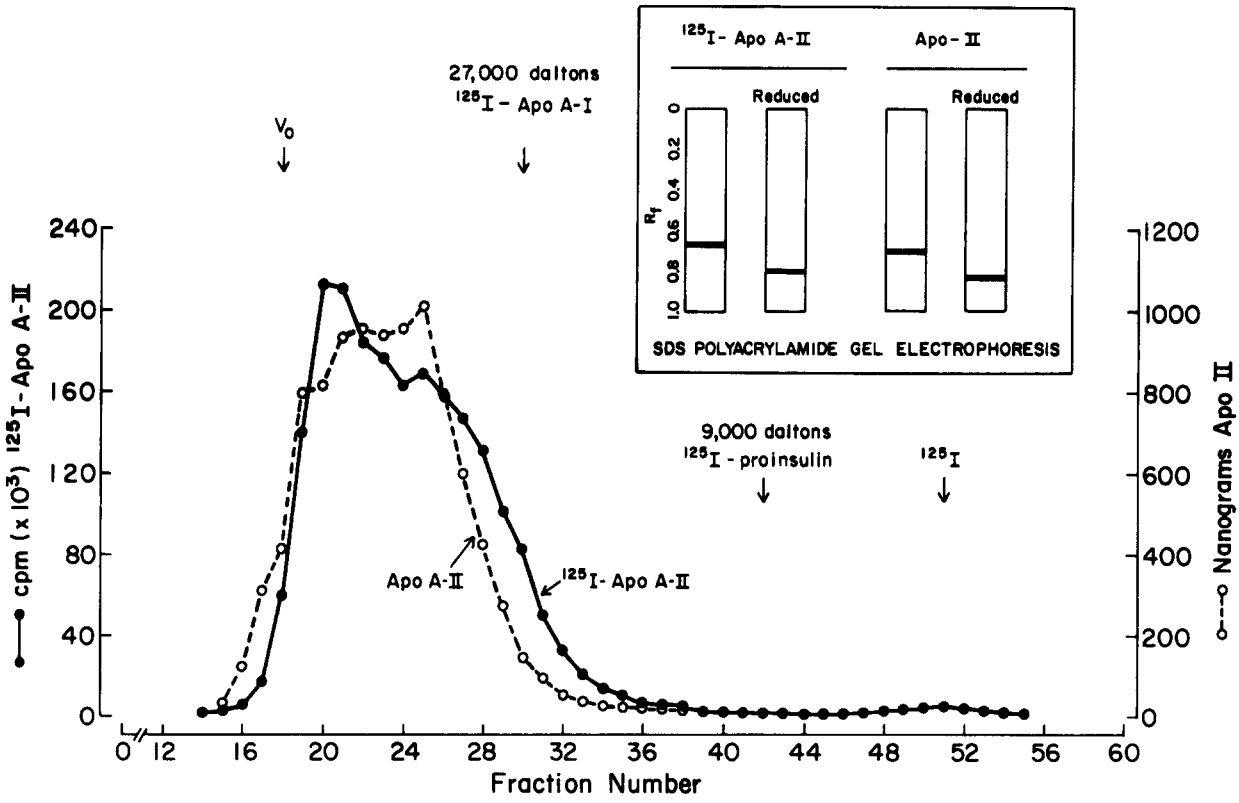

Fig. 2. Elution profiles of apo A-II (○ - - - ○) and ^{125}I-labeled apo A-II (● —— ●) Sephadex G-100 (1.25 × 50 cm) columns in borate/0.5% BSA buffer (pH 8.0). Vo, the void volume is marked according to the elution position of Blue Dextran; 27,000 and 9,000 molecular weight markers and the salt peak are arrowed. The profile of ^{125}I-labeled apo A-II was obtained from the radioactivity (cpm × 10^{-3}) in each fraction. The non-iodinated apo A-II elution pattern was obtained by assaying each fraction for its apo A-II content. The inset shows schematically the migration positions of apo A-II and ^{125}I-labeled apo A-II on polyacrylamide gel electrophoresis in 0.1% SDS with and without β mercaptoethanol.

fractionated ^{125}I-labeled apo A-II radioactivity profile on a Sephadex G-100 column (fractions 16–21).

Using this purified ^{125}I-labeled human apo A-II tracer and the anti-HDL antiserum at a titer of 1:50,000, the assay was sensitive to 0.3–0.4 ng (**Fig. 4**). Cross-reactivity with VLDL, A-I, or LDL was less than 0.2%. Both serum and HDL dilutions were consistently parallel to the standard curve. For the anti-HDL antiserum (G1) the slope of the standard curve (±2 SD) was −2.16 ± 0.1 and the mean (±SEM) of eight serum curves was −2.16 ± 0.03 in the same assay. Similarly using the anti-A-II antiserum (Ra IV), the slope of the standard was −1.89 ± 0.12 and the mean slope of three serum curves was −1.93 ± 0.03. Serum values using the anti-A-II antiserum were similar to those obtained with the G1 antiserum (e.g., for anti-A-II: 28 ± 2 mg/dl; for anti-HDL; 24 ± 2 mg/dl; n = 6).

Stability of standard preparations of apo A-II. Seven different apo A-II standards were used. Repeated freezing and thawing of apo A-II stored at −20°C resulted in a loss of immunoreactivity. Storage of aliquots (10–20 μg) of apo A-II at −60°C either in 0.1 M NH_4HCO_3, in 1.5 M guanidine HCl, or lyophilized, prevented deterioration of immunoreactivity for 6–12 months. Dilutions of apo A-II (1 μg/ml) for use as the standard are stable at 4°C for at least 4 weeks. Two apo A-II preparations that had been lyophilized were noted to display a progressive alteration in the elution profile after iodination. Using these tracers, serum values increased by 20%. Polyacrylamide gel electrophoresis of one of the preparations revealed an extra small molecular weight component.

Validity of the radioimmunoassay

Apo A-II values in 50 normal young men and women were 25 ± 1 mg/dl (means ± S.E.M.) and 26 ± 1 mg/dl, respectively. Apo A-II was fully recovered (95–112%) when added either in the form of purified apo A-II or as serum or HDL to several sera, HDL, and purified apo A-II samples. Compared to apo A-II concentrations in untreated sera, the values in 11

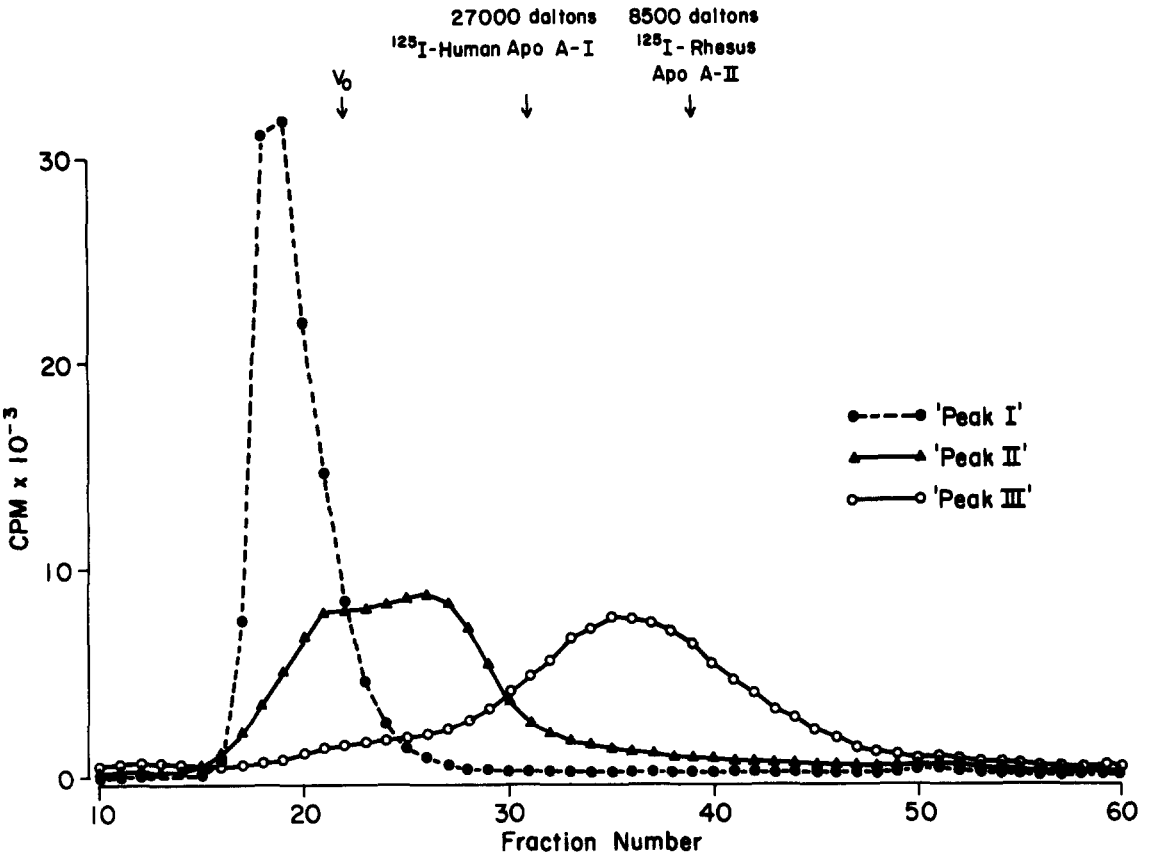

Fig. 3. Elution profiles of ^{125}I-labeled apo A-II subfractions applied to Sephadex G-100 (1.2 × 50 cm columns) in borate 0.5% BSA buffer (pH 8.0). Void volume, 27,000 and 8,500 molecular weight markers are arrowed. 'Peak I' material (● – – – ●) is taken from the first major peak (fractions 17 and 18) of freshly iodinated ^{125}I-labeled apo A-II chromatographed through Sephadex G-100; 'Peak II' (▲ —— ▲) is taken from the second major component (fractions 24–26) and 'Peak III' (○ —— ○) from a minor component (fractions 31–36) on the descending limb (see Fig. 2).

serum samples delipidated by ethanol–ether were 92 ± 4%, while the values in 30 sera delipidated with 8.4 M TMU in 8 M urea were 93 ± 3%.

Apo A-II values of three fresh sera diluted and assayed within several hours of venesection did not change upon the following manipulations: i) repeated freezing and thawing; ii) storage of whole sera at 4°C for 24 hr; iii) heating at 52°C for 3 hr; and finally, iv) storage at −20°C for one year. In addition, there was no difference in values measured in plasma collected in Na_2 EDTA.

Apo A-II in HDL

The A-II composition of HDL was measured in a number of preparations (**Table 2**). The effect of delipidation of HDL on the apo A-II immunoreactivity was tested by comparing the contribution of immunoreactive apo A-II to the total HDL protein measured by the method of Lowry et al. (15). Both ethanol–ether extraction and TMU-urea delipidation were assessed (**Table 3**). Neither procedure changed A-II immunoreactivity significantly, although ethanol–ether extraction led to substantial losses of protein.

Incubation of trace amounts of ^{125}I-labeled apo A-II with holo-HDL and HDL_3 samples before the delipidation procedure showed recoveries of 50% and 72% of the counts in the delipidated material. Most of the remaining counts were present in the organic phase.

Quantitation of apo A-II in HDL by polyacrylamide gel electrophoresis. The concentration of apo A-II in four different HDL_3 standard preparations, determined by quantitative SDS polyacrylamide gel electrophoresis and radioimmunoassay (**Table 4**), produced similar results. Reasonable agreement between the two methods was obtained in HDL samples with differing apo A-II compositions ($r = +0.73$) assessed by gel electrophoresis.

Quantitation of apo A-II in HDL subfractions prepared by ultracentrifugation. Less than 1% of the serum apo A-II was measured in the $d < 1.063$ g/ml fractions obtained during preparative ultracentrifugation, while approximately 3% was found in the $d > 1.21$ g/ml

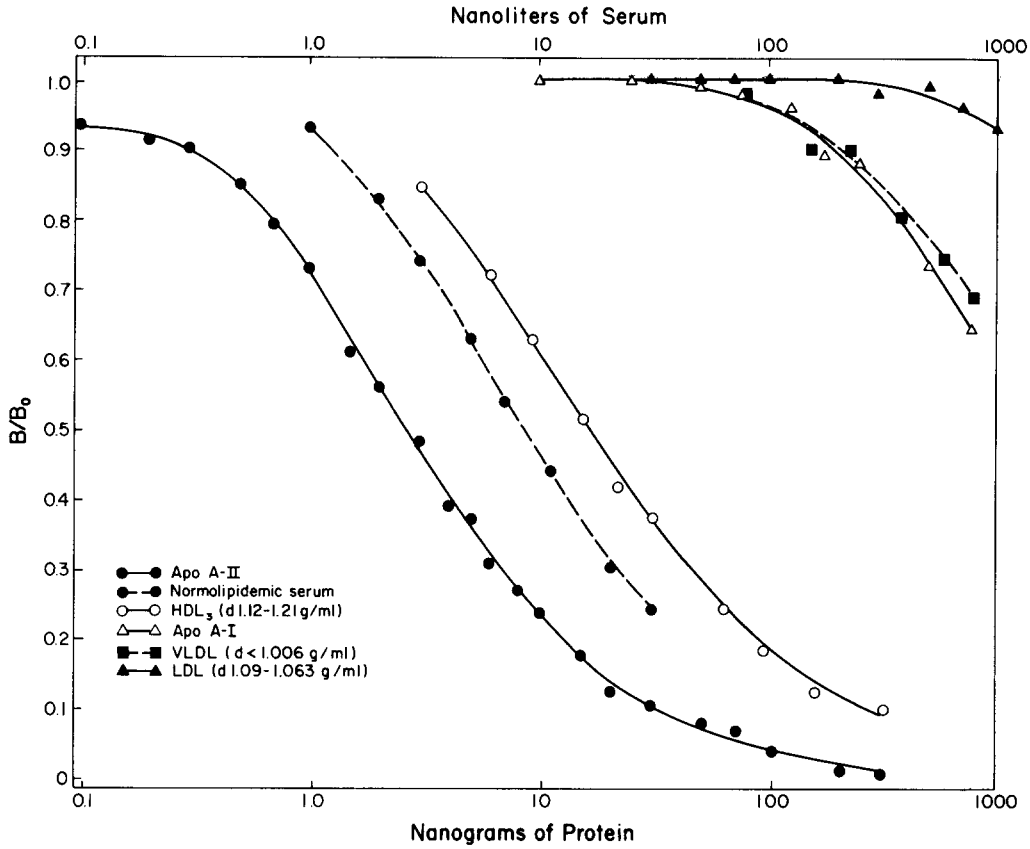

Fig. 4. Displacement of purified ^{125}I-labeled apo A-II (Peak I) from anti-HDL. Apo A-II (● —— ●); serum (● – – – ●); HDL_3 (○ —— ○); Apo A-I (△ —— △); VLDL d < 1.006 g/ml (■ – – – ■); LDL d 1.09–1.063 g/ml (▲ —— ▲). Protein concentrations were determined by the method of Lowry et al. (15).

infranate. The distribution of apo A-II and of apo A-I (7) in HDL subfractions was studied using the single-spin technique of gradient ultracentrifugation (20). An example of the distribution of apo A-II and apo A-I in the d < 1.21 g/ml fraction of serum is shown in **Fig. 5.** The major portion of apo A-II (78%) was distributed in the higher densities of the HDL class (HDL_3), while the lighter HDL fractions were relatively enriched in apo A-I.

DISCUSSION

Complex proteins such as the apolipoproteins pose major problems for immunological quantitation. First, in their native state, they are attached to lipids and this imparts a specific molecular conformation to the peptide. Second, interaction with lipid may 'mask' antigenic sites. Third, delipidation of the peptide, for the purpose of obtaining a pure standard, may produce a species with an altered structure that may influence its behavior in solution or on surfaces. Each of these phenomena may affect the interaction between the peptide and its antibodies.

Apo A-II has been a particularly difficult peptide to quantitate by immunological techniques, judging from the wide discrepancy in serum values obtained

TABLE 2. Apo A-II composition of HDL

HDL	n	Value	Range
d 1.063–1.21 g/ml	4	19 ± 4	10–28
d 1.063–1.12 g/ml	4	19 ± 3	14–28
d 1.12–1.21 g/ml	10	20 ± 3	11–33

Values are expressed as the percentages of the Lowry protein and are presented as the mean ± S.E.M.

TABLE 3. Effect of delipidation on HDL apo A-II

	Non-delipidated	TMU-Urea	Ethanol–Ether
HDL_3	11		6
HDL_3	22	19	19
HDL_3	12		16
HDL_2	28	26	24
HDL_{holo}	18	27	23

Results are expressed as the percentages of the Lowry protein. The methods of TMU-urea and ethanol–ether delipidation employed here are described in References 6 and 18.

by different investigators (8–12).[3] Although little is known of its conformation in HDL, interaction of A-II with lipid appears to require a specific molecular arrangement (23). Current evidence suggests that its antigenic sites are fully exposed (10, 11, 24). In addition, the purified delipidated peptide has distinctive physiochemical properties in solution, causing it to self-associate in a concentration-dependent manner which may be influenced by the nature of the aqueous solution (2), as well as by the presence of denaturating agents such as urea or guanidine HCl. Undoubtedly these properties of apo A-II have affected immunological methods and the purpose of these investigations was to study this problem further, using the radioimmunoassay technique.

Lack of immunological identity between serum or HDL and purified apo A-II

Important immunochemical differences between the purified apo A-II used as standard and tracer and apo A-II in serum or HDL were detected by the various antisera tested. This conclusion was derived from the observation that serum and HDL dilution curves were not parallel to that of the standard. Experiments with different purified apo A-II preparations, several HDL and apo A-II antisera, utilization of alternative buffers and protein carriers, inclusion of a detergent (Triton X-100), iodination by two different techniques, and, finally, attempts to modify HDL by heating failed to resolve the discrepancy. Reduced-and-alkylated apo A-II was tested as an alternative standard and tracer, but standard and serum curves were again not parallel. It is interesting to note that despite their close chemical and physical similarities, apo A-II competes poorly with reduced-and-alkylated apo A-II for antibody binding sites. In this respect non-reduced purified apo A-II more closely resembles serum apo A-II which also competes less efficiently with the reduced apo A-II tracer.

With the exception of Cheung and Albers (25), a lack of parallelism between standard and serum samples has not been described in published immunoassays for apo A-II. Using the radioimmunodiffusion method, these authors noted that the standard curve was not parallel to that of serum unless the Tris buffer used contained 9.4 M TMU in 8.0 M urea. There may be several reasons for the lack of comment by other investigators. *1*) It is a subtle discrepancy requiring dilutions over a 5–10 fold concentration range of serum or HDL to demonstrate consistently. For example, close examination of the data of Mao, Gotto, and Jackson (24) does suggest a lack of parallelism. Moreover, the extent of the discrepancy may vary both within and between assays. It is of importance to appreciate that serum values may decrease as much as 44% between B/Bo = 0.9 and B/Bo = 0.3. *2*) Different modes of preparation and storage of purified apo A-II and manipulation of serum or HDL may influence the differences. *3*) Antisera may vary in the extent to which differences between purified and serum apo A-II are detectable.

TABLE 4. Comparison of polyacrylamide gel electrophoresis with the radioimmunoassay as a means of quantitating the apo A-II in HDL_3

HDL_3 Preparation	I	II	III	IV
Polyacrylamide gel electrophoresis	26%	21%	20%	26%
Radioimmunoassay	22%	33%	29%	28%

Results are expressed as percent of Lowry protein. Quantitation of apo A-II by polyacrylamide gel electrophoresis is described in the text.

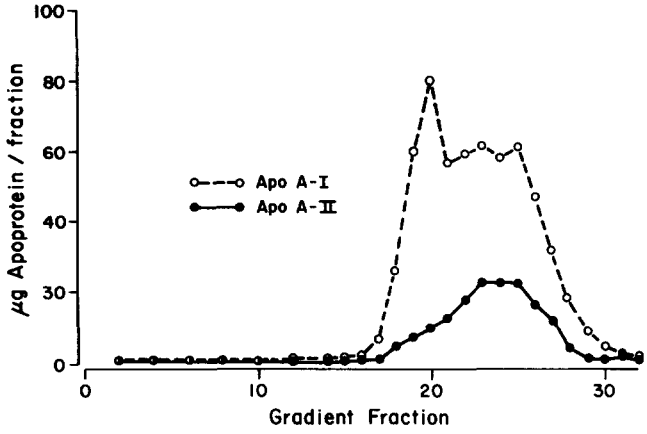

Fig. 5. Distribution of immunoassayable apo A-II (● —— ●) and apo A-I (○ – – – ○) after density gradient ultracentrifugation by the single-spin method (26) of the d < 1.21 fraction of serum (equivalent to 0.6 ml of serum) from a normolipidemic male. Apo A-I was measured by radioimmunoassay as previously described (7).

Gel filtration of apo A-II

Investigations into the possible reasons for the lack of identity between purified and native apo A-II led to the discovery of a hitherto unreported property of apo A-II. When a dilute solution of apo A-II (<15 μg/ml) is gel-filtered in an albumin-containing buffer on Sephadex G-100, it elutes with a broad profile originating in the void volume. At this low concentration, apo A-II is thought to be predominantly in monomeric form (2). However, the phsyicochemical properties of the apoprotein have not been studied at concentrations below 100 μg/ml. Possible explanations for this phenomenon include conformational changes at high dilution, interaction between apo A-II and the gel or buffer constituents, or some form of aggregation. In relation to the last-mentioned possi-

bility, neither avoidance of lyophilization nor prior incubation with guanidine HCl affected the elution profile. Recent observations on impurities in commercial bovine albumin preparations (26) may be important, although in preliminary experiments with crystalline albumin in the buffer, gel-filtered apo A-II still emerges in the void volume of a Sephadex G-100 column. Schonfeld et al. (10) noted that when iodinated apo A-II was applied to Sephadex G-75 columns equilibrated with a barbital–albumin buffer, it eluted in the region of the 24,000 molecular weight marker, while inclusion of Triton X-100 in the buffer caused the tracer to emerge near the void volume. Although current knowledge is insufficient to explain these findings, they illustrate the complex nature and behavior of apo A-II in solution.

It is likely that these properties of apo A-II seen on gel filtration contribute significantly to the immunochemical differences detected between purified apo A-II and apo A-II in serum or HDL. By selecting certain fractions from the broad radioactivity profile of gel-filtered iodinated apo A-II, a tracer is obtained that competes in an immunologically identical manner with both unfractionated standard and serum or HDL for antibody sites. Hence, parallel serum and standard displacement curves can now be obtained over a 10- to 20-fold concentration range, in the absence of any detergent.

Apo A-II in serum and HDL

Using the radioimmunoassay, our values in young non-obese men and women were 25 ± 1 and 26 ± 1 mg/dl, respectively. These values are lower than several reported values (8–11), but are similar to the electroimmunoassay results of Shepherd et al. (12) and Bronzert.[4] In view of the wide discrepancy in published serum values for apo A-II (25–81 mg/dl), it is logical to consider the possible causes underlying the variability in the absolute quantitation of apo A-II in serum. We found that: *1*) the mode of storage may affect the immunoreactivity of the apo A-II standard; *2*) a lack of parallelism between serum and standard curves may lead to different serum values according to whether the sample value is measured from the upper or lower part of the standard curve; and *3*) the specific immunologic technique employed may affect the serum values.

Electroimmunoassay and radial immunodiffusion techniques require that the ligand migrate electrophoretically or diffuse passively through a semi-solid agarose gel as it is immunoprecipitated. The electrophoretic and diffusion properties of delipidated apo A-II as compared to whole HDL have not been carefully studied. Thus using the 'rocket' technique, Schonfeld et al. (10) found unrealistically high HDL A-II values (based upon the expected apo A-II content of HDL) when read against a pure apo A-II standard. Substituting an HDL standard with an assigned apo A-II content (measured by column chromatographic methods) produced serum results (30–40 mg/dl) similar to those obtained by their radioimmunoassay which includes Triton X-100 in the buffer. It is interesting to note that Curry, Alaupovic, and Suenram (9), using the electroimmunoassay method based on a pure apo A-II standard, reported values at least twice those of others (80 mg/dl). Bronzert[4] has found that unless serum is delipidated, high apo A-II values are obtained by electroimmunoassay. Cheung and Albers (8) included TMU and urea in their radial immunodiffusion assay in order to obtain parallelism between pure apo A-II and serum or HDL immunoreactivities.

In an effort to corroborate our own radioimmunoassay results, we have utilized the method of Cheung and Albers (8). Omitting TMU and urea from this system gave serum values of approximately 100 mg/dl. These levels decreased to approximately 50 mg/dl by the inclusion of TMU and urea. This occurred because urea enhanced the reactivity of the apo A-II standard by an increase in the immunoprecipitate ring diameter, while TMU-urea reduced the reactivity of the serum samples. However, despite a close correlation between immunodiffusion and radioimmunoassay results ($r = +0.93$), the serum values obtained by this method remained significantly higher than those measured by our radioimmunoassay using the same standard and antiserum preparations.

Since these various methodological manipulations have been made somewhat empirically, investigators have attempted to test the validity of their particular technique by measuring the apo A-II content of HDL (8–12). We have found that the apo A-II contribution to HDL protein averages 20%. In contrast to the findings of Mao et al. (24), this was not influenced by delipidation nor was it affected by heating, thus confirming previous reports (8–11) that all of the antigenic sites of apo A-II appear to be exposed in HDL. We obtained similar results by quantitative polyacrylamide gel electrophoresis. Less than 5% of the apo A-II in serum was present in the $d < 1.063$ g/ml + $d > 1.21$ g/ml fractions obtained after ultracentrifugal isolation of serum lipoproteins. These results are consistent with the knowledge that the bulk of serum apo A-II is associated with HDL. The distribution of apo A-II after density gradient ultracentrifugation by the single spin technique confirmed these results. The majority of serum apo A-II was found in the HDL_3 subfraction as has been reported (25).

A number of other investigators have found a similar contribution of apo A-II to HDL (10–12) as we have and one would thus anticipate that their assays, as well as the one described by us, would give similar serum values of apo A-II. Nevertheless, evaluation of the results shows this is not the case. Shepherd et al. (12) found apo A-II comprised 15% of the HDL protein mass while serum values, by their method, were similar to those reported in this study. Assman et al. (11) noted that apo A-II contributed 18% to the protein mass in a single HDL sample, but serum values, by their technique, were nearly 50% higher than those we report. Similarly Schonfeld et al. (10), both by radioimmunoassay and column chromatography, estimated the apo A-II contribution to HDL protein to be approximately 20%, despite serum values almost 50% higher than we found. Lastly, Curry et al. (9) found that apo A-II comprised 30% of the HDL protein mass, yet serum levels, by their method, were more than three times higher than our values. Differences in the HDL preparations used may partly explain these inconsistencies, but it is necessary to consider that variations in assay methodology, antisera and standards, and alterations in isolated HDL as compared to that in whole serum may also be important.

In view of this analysis of the problem, it would seem difficult to definitively assign an absolute value for apo A-II in serum or HDL as quantitated by any of the existing immunological methods. Although this study has suggested some possible reasons for the discrepancies, further work will be needed to resolve all the problems. In the interim, it is necessary for each laboratory to rigorously establish its own normal values, with the expectation that relative differences will be consistent, although the absolute values may change as further work is done.

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